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RANGE OF MUTATION RATES

The Spontaneous Azaguanine-Resistant Mutants of Diploid Human Fibroblasts*

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Summary. The range of incidences of azaguanine-resistant colonies in cultures of fibroblasts from 16 unrelated humans was 0.4×10^{-6} to 19×10^{-6} and the mean value was 4.1×10^{-6}. A fluctuation test showed that most or all of the mutant colonies derived from mutations that occurred during *in vitro* proliferation of the fibroblasts and before exposure to azaguanine. The estimated rate of spontaneous mutation was 0.45×10^{-6} to 1.8×10^{-6} per cell generation. At least ten independent mutants, comprising two general classes, were studied. Class I mutants were a minority and resembled cells from boys having the Lesch-Nyhan syndrome: they had very little HG-PRT activity, showed maximum resistance to azaguanine and could not utilize hypoxanthine for growth. At least 90% of the mutants were in Class II: their apparent HG-PRT activities ranged between normal and Lesch-Nyhan amounts, they were partially sensitive to azaguanine and they could utilize hypoxanthine. Some Class II mutants resembled cells cultured from a family having an X-chromosomal mutant gene that does not cause the Lesch-Nyhan syndrome but does confer resistance to azaguanine, although the quantity of HG-PRT activity is apparently normal and hypoxanthine can be utilized. Electrophoretic differences between the HG-PRT activities of normal and mutant strains were not detected but other qualitative alterations were observed in some mutants.

Introduction

In the course of mutagenesis experiments with cultured human fibroblasts we detected a high incidence of 8-azaguanine-resistant (=AG^r) mutants that could utilize hypoxanthine for growth in untreated control cultures. The prototypes for diploid human cells that are resistant to AG are cells cultivated from males having the Lesch-Nyhan (= L-N) Syndrome (Nyhan, 1968). The HG-PRT (E.C.2.4.2.8, 1965) deficiency characteristic of these cells (Seegmiller *et al.*, 1967) has corollary expressions: I. as impaired ability to convert purine-analogue substrates to their toxic nucleotide derivatives and, II. as inability to utilize exogenous hypoxanthine for growth when the endogenous pathway of purine nucleotide biosynthesis is blocked with aminopterin or azaserine. It has sometimes been a premise of studying rates of mutation to AG-resistance with cultured cells that the mutants existing before a rate determination could be eliminated by prior cultivation of the cell population in HAT (Szybalski *et al.*, 1962) or THAG

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During the symposium, Dr. DeMars only gave a relatively short summary of his findings. However, I agreed with pleasure when he offered the complete manuscript for publication within this context (The Editor).

(Chu *et al.*, 1969) medium, which permit the proliferation of only those cells able to utilize exogenous hypoxanthine.

The first diploid AG^r mutant we isolated was, indeed, unable to utilize hypoxanthine (Albertini and DeMars, 1970) but such mutants have proven to be exceptional. This report presents evidence that most independent, spontaneous AG^r mutants of human fibroblasts can utilize hypoxanthine, indicating that pretreatments with HAT or THAG media may be ineffective in removing pre-existing mutants. Some of the mutants detected *in vitro* resemble cells from humans having a mutant allele of an X-chromosomal locus that affects HG-PRT but does not cause the Lesch-Nyhan Syndrome. We also describe a fluctuation test, which showed that most or all of the mutants we studied originated in mutations that preceded selection with AG and which provided an estimate of the rate of spontaneous mutation to AG-resistance.

Materials and Methods

Cells. Diploid fibroblasts from human skin. Strains 261, 361 and 363 were derived from forearm biopsies of adult females. All other cultures were derived from foreskins.

Media. F10 (Ham, 1963) modified by the omission of hypoxanthine was used throughout. FCS-F10 was composed of 85 volumes of F10 and 15 volumes of fetal calf serum mixed just before use. CS-F10 contained 85 volumes of F10 and 15 volumes of calf serum. CS-F10-AG contained 8×10^{-6} M 8-azaguanine. CS-F10-HAT contained aminopterin (2×10^{-7} M), thymidine (3×10^{-5} M) and hypoxanthine (3×10^{-5} M). Stock solutions of AG (8×10^{-4} M or 2×10^{-2} M) were prepared by dissolving the analogue in the minimum volume of 1 M NaOH, adding distilled water to the desired final volume and sterilizing by Millipore filtration. These solutions were stable at room temperature for months, unlike solutions of 6-thioguanine and 6-mercaptopurine, which deterioriated significantly under the same conditions.

Culture Vessels. Glass dishes 13 mm in diameter, called "P13's" (Bellco) were used for quantitative growth studies. Otherwise, plastic dishes (Falcon) that were 7 mm, 30 mm ("P30"), 60 mm ("P60") or 100 mm ("P100") were used.

Starting Cultures. Foreskins and other skin biopsies were minced after fatty tissue was trimmed. The pieces of each foreskin were immobilized under 25 mm diameter glass cover slips in 5 or 6 P30's containing FCS-F10, while only 1 P30 was used for smaller biopsies. The medium was replaced 2 or 3 times weekly and the primary outgrowths were usually subcultured within 2 or 3 weeks. At this stage, the cells from each P30 sufficed to inoculate one P60 or equivalent. The second subculture occurred about 1 week later, when the primary subcultures had become confluent. Cells from the second or third subcultures were used for the selection experiments described here and were also stored in liquid N_2. Cells that were thawed from liquid N_2 for use in some experiments increased at least 5-to-10-fold between thawing and use.

Trypsinization. The culture medium was removed from recently confluent monolayers (about 1.5×10^6 cells per P60) and was replaced with 1.5 ml of trypsin solution (GIBCO, 0.25%), which rinsed the monolayer and was, in turn, replaced with 1.5 ml of fresh trypsin. After 5-to-10 min of incubation at 37° 3.5 ml of FCS-F10 were added to each P60 and the cells were gently pipetted the minimum number of times needed to produce a monodisperse suspension. The residual trypsin had no detectable effects on the behavior of cells if it was finally diluted at least 10-fold in FCS-F10. Cells were counted with hemocytometers or with a Coulter Counter.

Stock cultures were inoculated with about 2-to-4×10^5 cells per P60, had medium replacements every second day and were subcultured at intervals of 7—10 days.

Selection Experiments. P60's were inoculated with 10^4 cells in FCS-F10. After 72 hrs the medium was replaced with CS-F10-AG, which was renewed thrice weekly for 21—28 days. The choices of inoculum size and AG concentration were made to maximize the recovery of mutants after preliminary studies (Albertini and DeMars, 1970, 1972). AG-resistant

colonies could often be detected microscopically by the 10th day of selection and after 21 days most colonies were visible to the naked eye when viewed with oblique lighting against a black background. At this stage colonies contained about 10^4—10^5 cells and were either isolated for propagation or were stained and counted.

Colony Counts. Colonies developing in non-selective medium were generally counted after 10—13 days, while mutant colonies that were selected in CS-F10-AG were counted after 21—28 days. The colonies were rinsed with 0.9% NaCl, fixed for at least 5 min with absolute methanol and stained with 2% methylene blue in 0.1 M citrate buffer at pH 6.0 for at least 5 min.

Growth Curves. Cells in FCS-F10 were inoculated into 13 mm diameter glass dishes (Bellco) or 7 mm diameter plastic wells (Falcon). After 15 to 24 hrs each culture was presented with experimental medium (0.5 ml for 13 mm dishes and 0.1 ml for 7 mm wells), which was renewed every second day. The cell populations in duplicate or triplicate cultures of each condition tested were determined with a Coulter Counter after 7 to 12 days, depending on the experiment.

Reverse Selection Cloning. We wanted to know if clones that initiated development in CS-F10-AG could continue growing in CS-F10-HAT. Plastic P60's were inoculated with 100 cells in FCS-F10, which was replaced with CS-F10-AG the following day. After 8—10 days 20 discrete colonies that contained 100—200 cells were located with the microscope and marked with a scribe mounted on the microscope nose piece. The medium was then replaced with CS-F10-HAT, which was renewed after 3 and 6 days. The sizes and mitotic activities of the colonies were noted on the 7th day.

HG-PRT Assays. Reaction mixtures (50 µl) contained Tris-HCl, pH 7.4 (10^{-2} M), $MgSO_4$ (5×10^{-3} M), PRPP (10^{-3} M), [8-^{14}C]-hypoxanthine (2×10^{-4} M, 3 mc/mmole) and amounts of cell protein ranging up to 50 µg. The 8-^{14}C hypoxanthine was obtained at about 50 mc/mmole from Schwartz. Incubations were at 37° C for 30—120 min and the reactions were stopped by the addition of 10 µl of 4 M formic acid. Non-radioactive hypoxanthine, inosine and inosinic acid (0.085 mmole each in a total volume of 50 µl) were added to each reaction mixture. samples (10 µl) were chromatographed (ascending) for 2.5 hrs at room temperature with 5% Na_2HPO_4 (Henderson *et al.*, 1968). Pieces of paper combining the inosine and inosinic acid spots were cut out and counted in a scintillation counter using a mixture of PPO (2,5-diphenyloxazole, 22.74 g), POPOP [1,4-bis-2-(5-phenyloxazoly) benzene, 0.037 g] and toluene (3.79 l). Some assays were performed with the Glass Cylinder Slide method (Russell and DeMars, 1967). Cell monolayers grown on microscope slides in glass cylinders were rinsed twice with 0.9% NaCl by filling up the cylinders. The cylinders were then filled with ice-cold acetone, which was removed after 60 sec at room temperature. The samples were air-dried for 10—20 min and then stored at —20 or —60° C. Determinations of protein content (Oyama and Eagle, 1956) and HG-PRT activity were made by adding reagents directly to the monolayers. The enzyme activity of stored samples was constant for at least 3 weeks.

Sonic extracts used in other assays were prepared by rinsing monolayers twice with 0.9% NaCl, scraping them into water (0.5—1.0 ml per P60) and rupturing the cells by a 45—60 sec treatment with a Medtron Ultrasonic Occillator. Dialysis was performed at 4° C with 1—3 ml samples in size 8/100 dialysis tubing against four one-liter changes of distilled water during 3—4 hrs.

Results

The Ubiquity of AG^r Variants. The incidences of AG^r colonies in cultures of fibroblasts from 15 unrelated males and 1 female are presented in Table 1. The range of incidences, 0.4-to-10×10^{-6}, is surprisingly narrow considering the opportunities for random events that could cause a large variance. Excepting the single, exceptionally high values for Strains 353 and 451, the mean incidence, weighted for the number of cells tested in making each estimate, is 4.1×10^{-6}. We believe the high values result from the use of only one or two P60 cultures as the sources of cells subjected to selection. If the inocula used in starting

Table 1. The Incidences of Azaguanine-resistant Colonies among Fibroblasts Cultured from 16 Unrelated Humans. The Colonies were Detected According to "Selection Experiments" in Materials and Methods

Strain	Cells Tested ($\times 10^{-6}$)	AG^r Clones	Incidence ($\times 10^{-6}$)
353	0.94	4	4.2
	1.00	5	5.0
	2.00	33	17.0
	0.88	4	5.5
	1.50	5	3.3
356	2.40	8	3.0
357	1.00	2	2.0
	1.00	10	10.0
359	2.00	11	5.5
370	2.40	1	0.4
371	2.40	18	7.5
376	1.00	1	1.0
377	1.88	7	4.1
407	2.00	17	8.5
261 (female)	1.00	1	1.0
426	1.10	8	7.3
450	1.00	3	3.0
451	1.00	19	19.0
452	1.00	2	2.0
453	1.00	1	1.0
454	1.00	1	1.0
		Weighted Mean:	4.1

these cultures sometimes contained one or more mutant cells the resultant mutant clones would be detected as many mutant colonies when the cells in the cultures were dispersed and challenged with AG. These observations and others indicate that it should be possible to obtain AG^r mutants from virtually any strain of diploid human fibroblasts. We have previously shown that doubly-marked male strains can be obtained by isolating AG^r clones from cultures that already possess a mutant gpd allele (Albertini and DeMars, 1970). Mutant 261.9 is an interesting extension of this ability to female cells that were already heterozygous for gpd alleles (DeMars, 1968).

Propagation of AG^r Variants. Clonal mutant cultures were established from colonies initially containing about 10^4—10^5 cells. The cells were isolated either by draining the culture vessels and trypsinizing the colony after surrounding it with a glass cylinder immobilized with sterile silicone stopcock lubricant (Dow Corning) or by scraping the colony into trypsin using a rubber policeman. The cells were dispersed by pipetting and distributed into several P30's or P60's, depending on the colony size. The first subculture was always made using FCS-F10, which was replaced with CS-F10-AG on the following day, if appropriate. We had noted that, at this stage, some mutants formed aggregates of cells that did not attach and spread well on the culture vessels if they were suspended in CS-F10-AG. Mutants from most of the strains listed in Table 1 were propagated

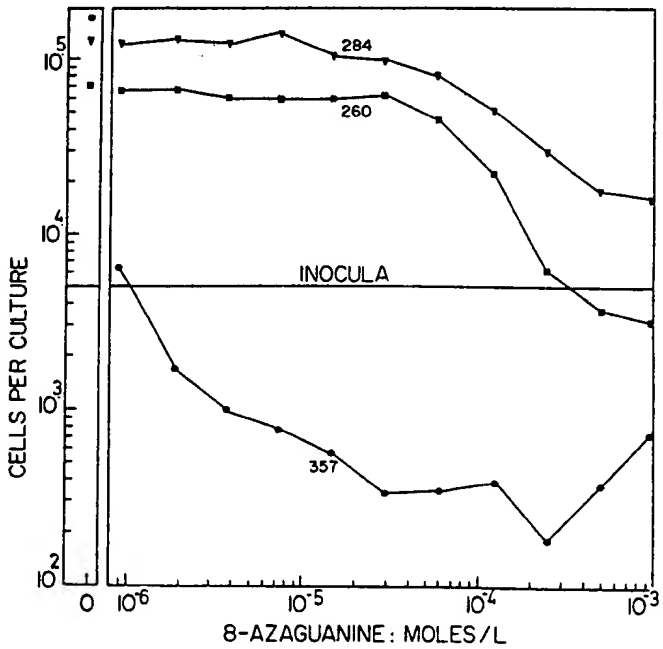

Fig. 1. *The growth of standard fibroblast strains as a function of 8-azaguanine concentration in CS-F10.* Strain 357, normal boy; Strain 284, boy with Lesch-Nyhan syndrome; Strain 260, boy with an X-chromosomal mutation that affected HG-PRT but did not cause Lesch-Nyhan syndrome. The cells were grown in P13 culture dishes and were counted 8 days after inoculation

in CS-F10-AG and characterized when their population sizes exceeded 10^6—10^7. Mutants from strains 261, 377, 407 and 426 were handled somewhat differently. Dishes were inoculated with either approximately 10^4 mutant cells each or about 10^3 mutant cells each. Some inocula of each size were propagated in FCS-F10 and others in CS-F10-AG. The populations generated from the smaller inocula were tested after they exceeded 10^6 cells and, generally, were derived from the pooled descendents of 10—100 subclones of the original mutant clone. These AG-grown "subclonal" populations could be regarded as effectively free of parental cells. Therefore, residual HG-PRT activity or ability to utilize hypoxanthine for growth, as described below, were attributes of AG^r cells.

FCS-F10-grown "subclonal" populations resulted from at least 10^4-to-10^5-fold increases in the absence of the selective agent. Persistent AG-resistance could then be regarded as a hereditary change rather than an adaptation that was maintained only in the presence of AG.

Populations of cells from AG-resistant colonies were characterized with regard to HG-PRT activity, AG-resistance and ability to use hypoxanthine for growth in comparison to standard mutant and normal cell strains.

Characterization of Standard Cell Strains: AG-resistance. Fig. 1 describes the growth of standard fibroblast strains in CS-F10 supplemented with various concentrations of AG. Strain 357 was typical of the many normal strains we have studied: growth was reduced at 10^{-6} M AG and the inhibition curve crossed the inoculum line at less than 3×10^{-6} M AG. Strain 284 was representative of 4 L-N-derived strains we studied: it was barely inhibited below 10^{-4} M AG and

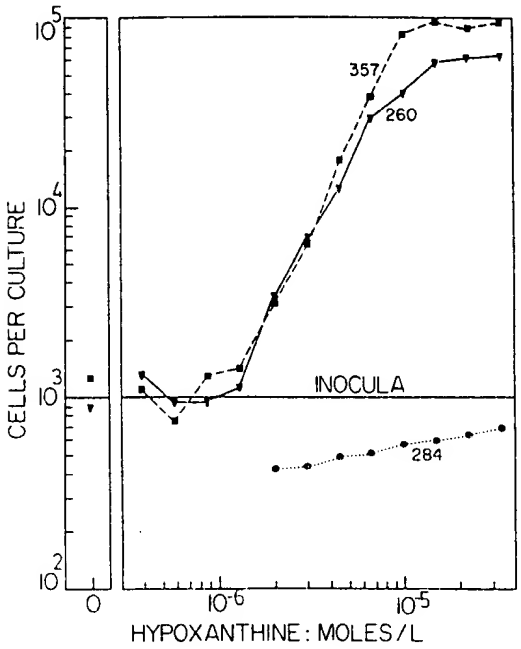

Fig. 2. *The growth of standard fibroblast strains as a function of hypoxanthine concentration in CS-F10-HAT*. The cell strains were the same as those used in Fig. 1. The cells were grown in P13's and counted 9 days after inoculation

the inhibition curve did not reach the inoculum line at 10^{-3} M AG. Strain 260 was derived from a hyperuricemic male who doesn't have the Lesch-Nyhan Syndrome (Benke and Herrick, 1972) but who evidently has an X-chromosomal mutant gene that affects his HG-PRT activity (see below). It is presented as a "standard" strain here because many spontaneous mutants recovered from normal strains *in vitro* resemble it more closely than they do standard L-N cells. The growth of Strain 260 fibroblasts was partially inhibited at 10^{-4} M AG and was completely inhibited at 10^{-3} M AG.

Use of Hypoxanthine for Growth. Fig. 2 describes the growth of the standard strains in CS-F10-HAT containing different concentrations of hypoxanthine. Strain 357 was again typical of normal strains: growth response above 10^{-6} M hypoxanthine, rising to a maximum above 10^{-5} M. A typical L-N response was shown by Strain 284: no net increase at any tested concentration of hypoxanthine. The ability of Strain 260 to grow as well as the normal strain in different concentrations of hypoxanthine was unexpected in cells that were highly resistant to AG. Resistance to aminopterin was not the explanation, since the cells failed to grow unless hypoxanthine was provided. We think impaired cellular permeability to hypoxanthine and its analogues might have been manifested by a shift in the hypoxanthine dependence curve toward the higher concentrations but that was not observed, either.

Characterization of In Vitro Mutants: HG-PRT Activity. The HG-PRT activities of 18 mutant colonies derived from 6 cell strains are shown in Fig. 3. One mutant, 261.9, had activity similar to that of its parent strain, but all the others had activities that were 30% or less of parental values. Some mutants had activities as low as those found in L-N Strain No. 252. Clearly, AG-resistance in spontaneous

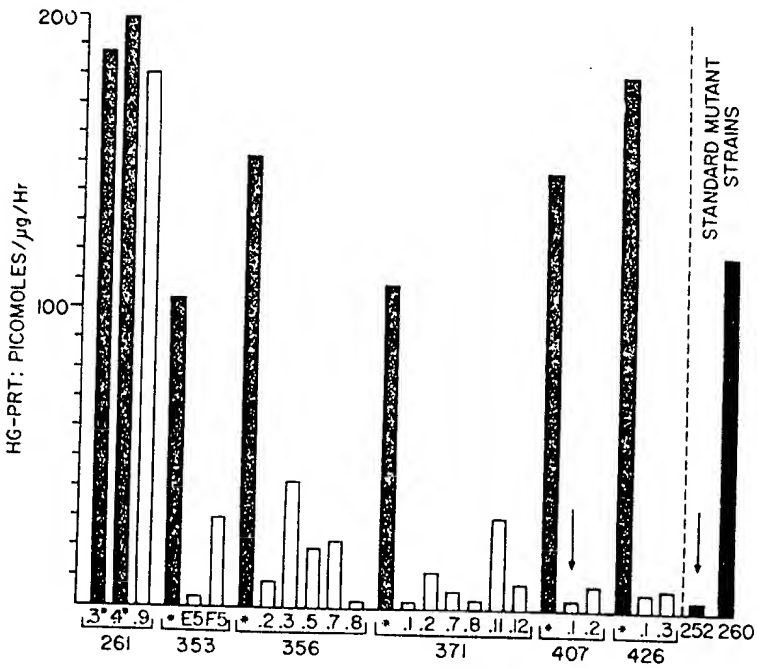

Fig. 3. *The HG-PRT activities of standard azaguanine-resistant strains, normal parental strains (*) and their derived azaguanine-resistant mutants (unfilled bars).* Averages of at least 3 determinations using the Glass Cylinder Slide method are shown except for the activity of 371*, which was determined with a sonic extract. Excepting mutant 261.9, the range of mutant values never overlapped the normal range. All strains except those indicated by arrows could use hypoxanthine for growth

mutants of cultured human fibroblasts is almost always associated with a significant reduction in HG-PRT activity as assayed under conditions optimized for the normal enzyme. The derivation of separate clones with distinctly different activities from the same parental strain suggests they originated from different mutations. Other differences between mutants 407.1 and 407.2, in particular, indicate that they are independent (see below). A minimum estimate of the number of independent mutants included in this series is 10 if we count mutants 371.1, 371.11, 356.3, 356.8, 353.E5, 353.F5, 261.9, 407.1, 407.2 and 426.1 as being independent because they occurred in cultures from different humans, because of distinctly different HG-PRT activities or because of different growth properties. The mutation rate (see below) is easily high enough to permit the detection of independent mutants in populations of 10^6 cells or more.

AG-resistance and Hypoxanthine Utilization of Spontaneous Mutants. The AG-challenged growth responses of mutants 407.1, 407.2, 261.9 and of two non-AG-selected clones of Strain 261 are compared in Fig. 4. Normal clones 261.3 and 261.4 showed typical sensitivity to AG and mutant 407.1, which has very low HG-PRT activity (Fig. 3), was uninhibited at 10^{-4} M AG. Mutants 261.9 and 407.2, which have high and low-intermediate activities, respectively, were partially inhibited at 10^{-4} M AG.

The growth responses of these clones as a function of hypoxanthine concentration in CS-F10-HAT are compared in Fig. 5. The normal clones (261.3, 261.4) responded maximally to concentrations of 10^{-5} M or more, as did mutants 261.9

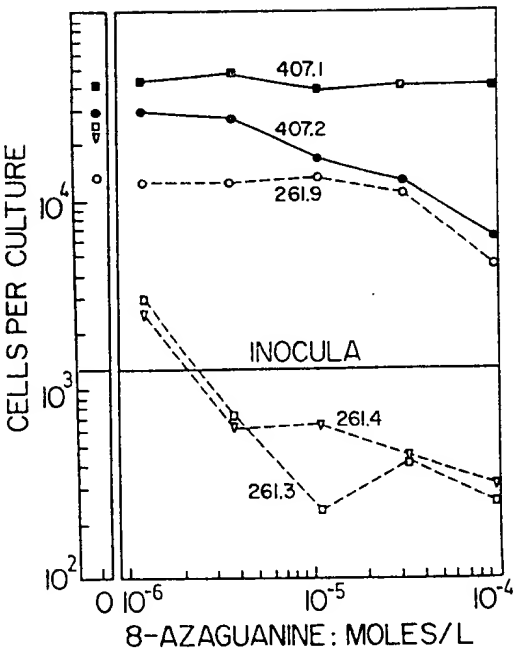

Fig. 4

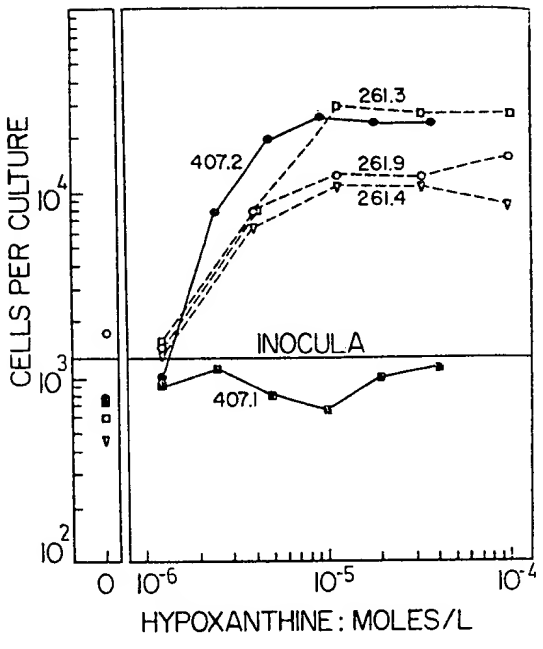

Fig. 5

Fig. 4. *The growth of 2 normal clones (261.3, 261.4) and 3 azaguanine-resistant mutants (407.1, 407.2, 261.9) as a function of azaguanine concentration in CS-F10.* The cells were grown in 7 mm diameter plastic wells and were counted 12 days after inoculation

Fig. 5. *The growth of 2 normal clones (261.3, 261.4) and 3 azaguanine-resistant mutants (407.1, 407.2, 261.9) as a function of hypoxanthine concentration in CS-F10-HAT.* The cells were grown in 7 mm diameter plastic wells and were counted 12 days after inoculation

and 407.2. In contrast, mutant 407.1 failed to increase at any tested concentration of hypoxanthine.

We used Reverse Selection Cloning (Materials and Methods) to directly demonstrate that AG-resistance and HAT-growth were attributes of the same cells and not a mixed cell population. All clones of mutants 407.2 and 261.9, which had achieved 100—200 cell size in CS-F10-AG maintained growth in CS-F10-HAT. In contrast, the growth of 407.1 clones initiated in CS-F10-AG was quickly arrested when they were transferred to CS-F10-HAT.

Our observations of HG-PRT activity, AG-resistance and HAT-growth can be summarized as follows: I. Mutant 407.1 resembles standard L-N cells in its relatively great resistance to AG, its inability to utilize hypoxanthine for growth and its scant (but real!) HG-PRT activity. Three other clones, isolated from Strain 407 in the same experiment had similar properties. Since this type of mutant has only been recovered from Strain 407, so far, we think the 4 clones we tested were descendants of a common ancestor. We were able to recover them because the cultures used in this study were not grown in CS-F10-HAT prior to selection for mutants. II. Mutant 261.9 resembles standard Strain 260 in its partial sensitivity to 10^{-4} M AG, its ability to utilize hypoxanthine and its apparently normal amount of HG-PRT activity. This is the only mutant we have studied that has activity fully in the normal range. III. Mutant 407.2 exemplifies

the great majority of spontaneous AG-resistant mutants in its partial sensitivity to AG, its ability to utilize hypoxantine for growth and in having low-to-intermediate HG-PRT activity. At least 90% of the independent clones studied in this sample were of this general type and unlike standard L-N cells.

The Genetic Nature of AG-resistant Variants: Estimation of the Spontaneous Mutation Rate. Sub-clonal populations of 261.9, 407.1 and 407.2 were prepared in FCS-F10 as described under *Propagation of AG^r Variants* and were challenged with AG. In each case we observed AG-resistance that had persisted during at least a 10^4-to-10^5-fold increase in the absence of the selective agent (Fig. 4). This is evidence that AG-resistance results from a hereditary change and not from an adaptation that is maintained only in the presence of AG. Furthermore, a fluctuation test (Luria *et al.*, 1943) has demonstrated that many and perhaps all, of the AG^r variants we recovered originated before the parental cells were exposed to AG.

Table 2 shows that the initial incidence of AG-resistant cells in Strain 426 was 7.3×10^{-6}. Since the effective starting population of cells in the 98 Set II cultures was 5.5×10^3 the chance that a mutant, clone-forming cell was initially present was about 0.04 and the chance that an individual Set II culture was inoculated with a mutant cell was about 4×10^{-4}. The 32 Set II cultures that yielded one or more mutant colonies evinced the occurrence of new mutations that originated either preadaptively, during non-selective outgrowth of the cells, or adaptively, in response to the subsequent challenge with AG.

A prediction based on the adaptive origin of AG-resistant variants is a random (Poisson) distribution of mutant colonies among the 294 P100's in which selection occurred. The distribution is non-random: I. The average number of mutant colonies was about 0.01 per P100 but 11 P100's had more than one colony. However, some of these dishes could have resulted from satellite colony formation by cells dislodged from mutant colonies during manipulation of the cultures. II. In 11 cases 2 or 3 P100's inoculated with the cells derived from a single Set II culture contained at least one mutant colony. It is more difficult to explain clustering of mutants in multiple dishes derived from the same culture by satellite colony formation and we conclude that these multi-dish clusters derive from mutants that proliferated before selection. Assuming randomness we would expect at least 11 of the Set II cultures that yielded just one mutant colony to have resulted from preadaptive mutations. This suggests that at least a majority, if not all, of the mutant colonies we detected originated in mutations that occurred before the introduction of AG.

If each recovery of one or more mutant colonies from a Set II culture identified a single, spontaneous mutation, the average number of mutations, x, could be calculated by solving $Po = e^{-x}$, where Po is the fraction of all Set II cultures that failed to yield a mutant colony. We chose the null-fraction method for estimating x in order to avoid errors in the numbers of mutant colonies per culture that might result from satellite colonies. This fraction was 0.67 and x was 0.395 per Set II culture, indicating that 38.7 independent mutations had occurred in the 98 Set II cultures. These mutations occurred in the course of a number of cell generations, $\bar{N}$, given by

$$\bar{N} = N\text{-}No/\ln 2 = 2.14 \times 10^7.$$

Table 2. *A Fluctuation Test Estimating the Spontaneous Rate of Mutation to AG-resistance in Human Fibroblasts.* Two sets of cultures were started simultaneously from two pooled P60 cultures of Strain 426. Set I consisted of 1.1×10^6 cells in FCS-F10 distributed in 100 P60's. Selection with CS-F10-AG was begun 72 hrs later and was terminated on day 22 after inoculation, when the AG-resistant colonies were counted (Entry 2). Set II cultures were started with 17,402 cells in FCS-F10 distributed among 113 P60's. The non-selective medium was replaced on days 5, 10 and 12. Colony counts were made with 10 Set II cultures on day 11 (Entry 3). On day 13 the cell populations in 5 Set II cultures were individually determined. The average was 1.35×10^5 cells per culture. The 5 cell suspensions were then pooled and diluted for a determination of the cloning efficiency of Set II cells in FCS-F10 (Entry 6). During the ensuing four hours 98 Set II cultures were individually trypsinized and the cells from each culture in FCS-F10 were distributed into 3 P100's. We estimated that the average cell number of these Set II cultures increased to 1.5×10^{-5} cells during this interval (Entry 5). CS-F10-AG was added to the Set II subcultures 72 hrs after inoculation. The numbers of mutant colonies were determined after 28 days of selection (Entry 7)

1. Parent strain: 426
2. Initial frequency of AG-resistant cells: 7.3×10^{-6} (8 colonies were recovered from 1.1×10^6 cells tested in Set I cultures).
3. Cloning efficiency of cells initiating Set II cultures: 0.37 (564 colonies formed in FCS-F10 in 10 P60's inoculated with 1540 cells).
4. N_0, The effective number of cells initiating 98 Set II cultures: 5527 (i.e. 98 P60's × 154 cells/P60 × 0.37).
5. N, The number of cells formed by non-selective growth in 98 Set II cultures: 1.48×10^7 cells.
6. Cloning efficiency of cells formed in Set II cultures: 0.31 (345 colonies formed in FCS-F10 in 9 P60's inoculated with 1125 cells from 5 pooled Set II cultures).
7. Numbers of AG-resistant mutant colonies in 98 Set II cultures.

Mutant Colonies	Set II Cultures	Mutant Colonies	Set II Cultures
0	66	5	1
1	21	6	0
2	4	7	1
3	4	8	1
4	0	>8	0

8. Mutation Rate: a) 1.8×10^{-6} per cell generation
   b) 4.5×10^{-7} per cell generation
   (see text for details of calculation)

The mutation rate, m, was derived from the expression:

m = number of mutations/number of cell generations = $m/\bar{N}$ = 1.81×10^{-6} per cell generation.

There are several potential sources of error in this kind of estimate and two of them are discussed here:

I. Inefficient recovery of mutants due to the need for segregation and phenotypic lags. We can't define, at this time, the interval needed for selectable phenotypic expression after a mutation has occurred. Incomplete penetrance could lead to underestimation of the mutation rate and this is the reason for including a 3-day interval in non-selective medium between inoculation of cultures and the

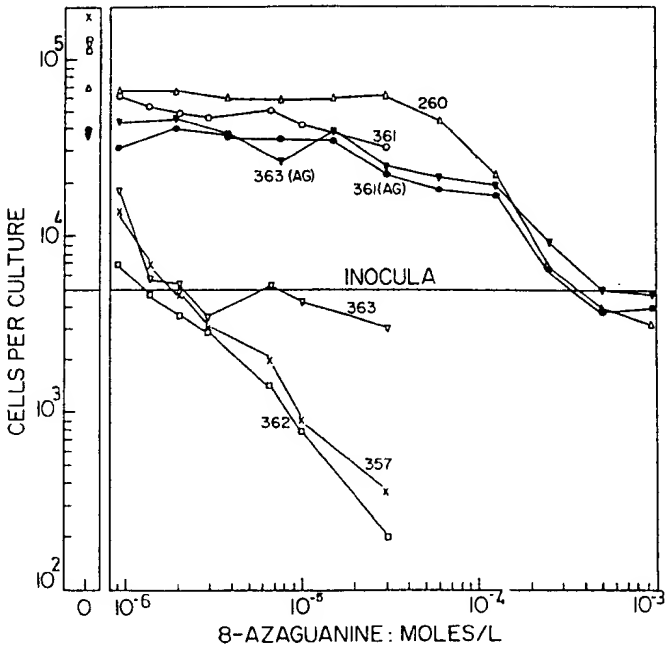

Fig. 6. *Azaguanine resistance resulting from an inherited X-chromosomal mutation that does not cause the Lesch-Nyhan syndrome.* Strain 357, normal boy; Strain 260, affected, hyperuricemic boy; Strain 361, mother of No. 260; Strain 362, father of No. 260; Strain 363, sister of No. 260; Strain 361 (AG), the phenotypically "mutant" heterozygous cells isolated from No. 361 by selective growth in CS-F10-AG; Strain 363 (AG), the phenotypically "mutant" cells isolated from No. 363. The data are from two different experiments, one with 260 and the second with the other strains. The cells were grown in P13's and were counted 8 days after inoculation

beginning of selection. II. Additional mutations might occur during the initial 3-day lag between dispersal of cells in Set II cultures and the beginning of selection. Cells usually increase about 4-fold during this interval. If selectable, these mutations inflate the estimate of the number of mutation events relative to the number of cells in the inoculum. Assuming a 4-fold increase during the 3-day lag in this experiment, a revised estimate of the mutation rate would be:

$$m = 38.7/8.55 \times 10^7 = 4.53 \times 10^{-7} \text{ per cell generation.}$$

We should be able to partially correct for the influence of "lag" phenomena in future experiments.

Evidence for an X-chromosomal Location of the Mutation in Standard Strain 260. The traits of many spontaneous, *in vitro* mutants, especially 261.9, mimic those of standard Strain 260. Two kinds of evidence indicate the mutant gene present in No. 260 is X-chromosomal and affects HG-PRT. First, there are two phenotypic classes of fibroblast clones in the mother and sister, but not the father, of the proband. The "normal" class is AG-sensitive and the other, "mutant", class is AG-resistant. Second, the HG-PRT activities of Strain 260 and of the AG-resistant fibroblasts derived from his mother and sister are qualitatively abnormal.

Fig. 6 depicts AG-challenged growth of strains derived from the proband, his mother, father and sister. The parental culture (No. 362) was normally sensitive in comparison to a standard normal strain (No. 357) but AG-resistant components

Table 3. *The Incidences of Azaguanine-resistant Colonies among Fibroblasts Cultured from Females who are Heterozygous for an X-chromosomal Mutation that Causes Overproduction of Uric Acid but not the Lesch-Nyhan Syndrome.* Strain 361, mother of No. 260; Strain 363, sister of No. 260. Cells were inoculated in FCS-F10, which was replaced with CS-F10 or CS-F10-AG on the following day. The media were renewed after 3, 7, 11 and 15 days. Colonies were counted 20 days after inoculation. C.E.: Cloning efficiency

Cell Strain	Exper. No.	CS-F10			CS-F10-AG			"Mutants"-C.E.$_b$/C.E.$_a$
		Inoculum	Clones	C.E.$_a$	Inoculum	Clones	C.E.$_b$	
361	I	255 cells in 3 P60's	53	0.208	595 cells in 7 P60's	87	0.146	0.702
	II	1050 cells in 7 P60's	214	0.204	1050 cells in 7 P60's	126	0.120	0.588
362	I	289 cells in 3 P60's	55	0.190	673 cells in 7 P60's	25	0.037	0.195
	II	833 cells in 5 P60's	214	0.257	5000 cells in 10 P60's	205	0.041	0.160

were evident in cultures derived from the mother (No. 361) and a sister (No. 363). We twice estimated the fraction of all cells that were AG-resistant by counting clones that formed in CS-F10 and in CS-F10-AG. Table 3 shows that the average incidence of AG-resistant clones in the maternal culture (No. 361) was 0.66 and was 0.18 in the culture (No. 363) of the sister's cells. As a part of Experiment II, 20 clones of Strains 260, 361 and 363 were subjected to Reverse Selection Cloning (Materials and Methods). In each strain, the 20 clones that had initiated growth in CS-F10-AG then maintained growth in CS-F10-HAT, demonstrating that AG-resistance and HAT-growth were properties of the same cells and that Strain 260 was not a mosaic of normal and L-N-type cells.

The greater incidence of AG-resistant clones in Strain 361 than in Strain 363 agrees with expectation based on their AG-challenged growth but, for both strains, the incidence of "mutant" clones is greater than expectation based on Fig. 6 and on the following assumptions: I. The AG-resistant female cells are as resistant as Strain 260 cells; II. The AG-resistant cells increased in CS-F10-AG as much, relative to their initial numbers, as did the total population in the absence of AG. The AG-resistant fractions estimated in this way were 0.37 for Strain 361 and 0.04 for Strain 363. We attributed these discordances between clones and populations to "metabolic cooperation" (Subak-Sharpe *et al.*, 1969; Friedman *et al.*, 1968), which causes cells that are genotypically AG-resistant to become phenotypically AG-sensitive after contact with normal cells (Albertini and DeMars, 1970, 1972). The inoculum density in our growth inhibition experiments (Fig. 6) was great enough to permit numerous cell-cell contacts and, in other experiments, to reduce the recoverability of L-N cells admixed with normal cells in CS-F10-AG. We think this interpretation is correct because Table 4 shows that Strain 260, despite its distinct differences from L-N strains, is also made sensitive to AG in the presence of sufficiently high densities of normal cells (Strain 357), and to about the same extent as L-N cells (Albertini and DeMars, 1970).

Table 4. *The Efficiency of Recovery of Strain 260 Colonies during Selection with CS-F10-AG in the Presence of Normal Cells.* Each P60 was inoculated with an average of 150 cells of AG-resistant Standard Strain 260 mixed with various numbers of normal cells (Strain 357) in FCS-F10. 72 hrs later the medium was replaced with CS-F10-AG, which was renewed at 2 day intervals. Colonies were fixed, stained and counted between 13 and 21 days after inoculation. The different periods of growth were chosen to avert inaccurate counts resulting from confluence of colonies and to permit maximum recovery of slow-growing colonies

Number of Strain 357 Cells per P60	Number of P60's	Number of Strain 260 Colonies = N	N/No
41.6×10^4	4	0	< 0.010
20.8×10^4	4	0	0.010
10.4×10^4	4	0	0.010
5.2×10^4	4	13	0.061
2.6×10^4	4	38	0.179
1.3×10^4	4	105	0.490
0	4	213 = No	1.000

Table 5. *NaF Inhibition of HG-PRT Activity in Sonic Extracts of Normal and Mutant Strains.* Standard assays, except for the addition of NaF, were performed with 60 min incubations. See caption to Fig. 6 for explanation of cell strains

Strain	CPM		a/b
	M/20 NaF = a	no NaF = b	
362	356	493	0.72
260	16	384	0.04
362(3):260(1)	228	586	0.39
362(1):260(1)	155	471	0.33
362(1):260(3)	64	484	0.13
361	289	1117	0.26
361 (AG)	52	658	0.08
363	445	950	0.47
363 (AG)	81	915	0.09

The "mutant" heterozygous cells can be conveniently separated from their "normal" sisters in uncloned populations by applying selection in CS-F10-AG to inocula of about 10^3 cells per P60. These AG-fractionated, heterozygous cell populations then have the properties of male, mutant cells (DeMars, 1972). Curves 361(AG) and 363(AG) of Fig. 6 show that they are just as resistant to AG as is Strain 260. They also have the characteristically altered HG-PRT activity found in Strain 260.

Using the study of bacterial AG-resistant mutants as a guide (Adye and Gots, 1966), Dr. Paul Benke (personal communication) has found that the red cell HG-PRT activity in No. 260 is more sensitive to NaF and has a lower affinity for PRPP than the normal activity. These differences have been confirmed by us with cultured fibroblasts and are illustrated here with data concerning fluoride-inhibition. We studied inhibition over the range of M/80-to-M/10 NaF and found that M/20 permitted the sharpest discrimination between Strain 260 and normal strains. Table 5 shows that fractional residual HG-PRT activity at M/20 NaF was 0.04 for Strain 260 and 0.72 for Strain 362, derived from his father. Various

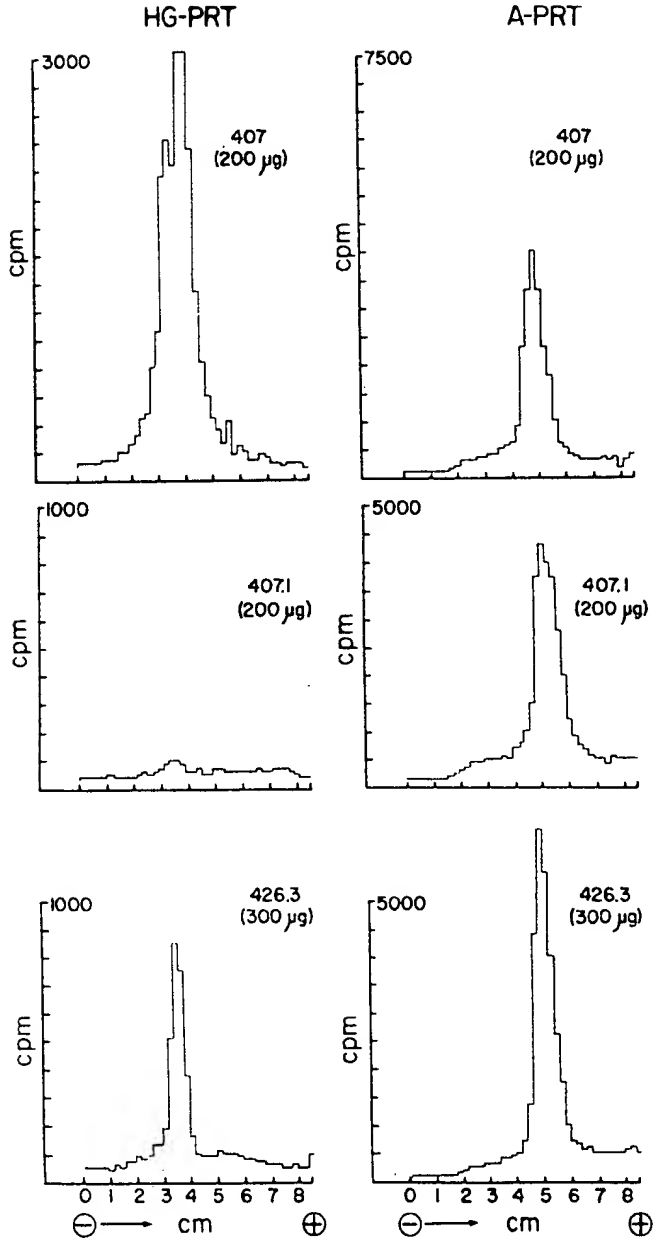

Fig. 7. *Electrophoresis of HG-PRT and A-PRT (adenine phosphoribosyltransferase) from 1 normal strain (407) and 2 azaguanine-resistant mutants (407.1, 426.3) in polyacrylamide.* Monolayers of cultured fibroblasts were rinsed twice with 0.9% Na Cl and scraped into saline. The cells were washed 3 times with saline using centrifugation at about 3000 × g for 5 min. The pellet was taken up in an equal volume of double-distilled water and the cells were lysed by 3 cycles of rapid freezing and thawing using an acetone-dry ice bath. The lysates were centrifuged at 25.000 × g for 20 min and the supernatant fluids were kept on ice prior to assay. — — For the separation of the A-PRT and HG-PRT we used the buffer system described by Rodbard and Chrambach (1971), consisting of: separation gel buffer (0.3750 M tris, 0.06 M hydrochloric acid), stacking gel buffer (0.0587 M tris, 0.0320 M phoshoric acid), cathodic buffer (0.0547 M tris, 0.0546 M glycine), anodic buffer (0.0625 M hydrochloric acid). Stacking gel: 0.6×1.0 cm, 2.5% total acrylamide concentration, 16% crosslinking. Separation gel: 0.6×8.5 cm, 7.5% total acrylamide concentration, 2.66% crosslinking. We used 100—300 μg of protein per gel. Electrophoresis was performed at a constant current of 1.5 mA

mixtures of extracts of the two strains showed intermediate degrees of inhibition. Heterozygous Strain 361 behaved like a mixture containing 50—75% "mutant" cells and the AG-resistant cell population extracted from it was almost as sensitive to M/20 NaF as was the standard mutant Strain 260. The NaF inhibition of HG-PRT activity shown by the other heterozygous strain, No. 363, corresponded to that of a mixture containing 50—75% "normal" cells. Again, the cell population obtained by selection with AG [No. 363 (AG)] was inhibited to a degree resembling that shown by the standard Strain 260. These determinations were made using cells disrupted with ultrasound but the greater NaF sensitivity of HG-PRT in Strain 260 in comparison to Strain 362 is clearly demonstrable using acetone-fixed, glass cylinder slide cultures and this may prove to be a useful way of classifying some AG^r mutants.

The occurrence of two phenotypic classes of clones, "normal" and "mutant", in the mother and sister of No. 260 but not in his father is good evidence that the mutant gene being transmitted in this family is on the X chromosome. Such clonal, single-allele expression in cultured female cells has been observed for the X-chromosomal mutant genes responsible for the L-N syndrome (Rosenbloom *et al.*, 1967; Salzmann *et al.*, 1968; Migeon *et al.*, 1968). The discovery of fluoride sensitive and PRPP dependent HG-PRT activity in the red cells of No. 260 and our demonstration that similarly altered HG-PRT activity is found in the AG-resistant cells of Strain 260 and of two presumably heterozygous females suggests that the mutation has affected the structure of the HG-PRT enzyme and may have occurred in the same structural gene in which L-N mutations have occurred. HG-PRT purified from normal and from mutant cells must be compared before this can be proven. The observations made with No. 260 and his family show that the traits of AG-resistance, HAT-growth and substantial HG-PRT activity, which we found associated in many spontaneous mutants *in vitro*, are also the phenotypic expressions of mutant alleles inherited *in vivo*. Similar concordance has been observed between the traits of spontaneous mutants *in vitro*, such as clone 407.1, and cells cultured from boys with the L-N syndrome. Nevertheless, large scale genetic and enzymological study of independent mutants is necessary in order to determine in which genes the mutations occur. The search for structural alterations in HG-PRT is one approach to making this determination and we present here some preliminary observations of HG-PRT activity in normal cells and mutant derivatives.

Preliminary Observations of HG-PRT Activity in Mutant 407.1: Electrophoretic Mobility. Fig. 7 displays the electropherograms of HG-PRT derived from a normal Strain 407 and from mutant clones 407.1 and 426.3. Our interpretation of these results and others made with a total of 12 mutants from 6 different strains of cells

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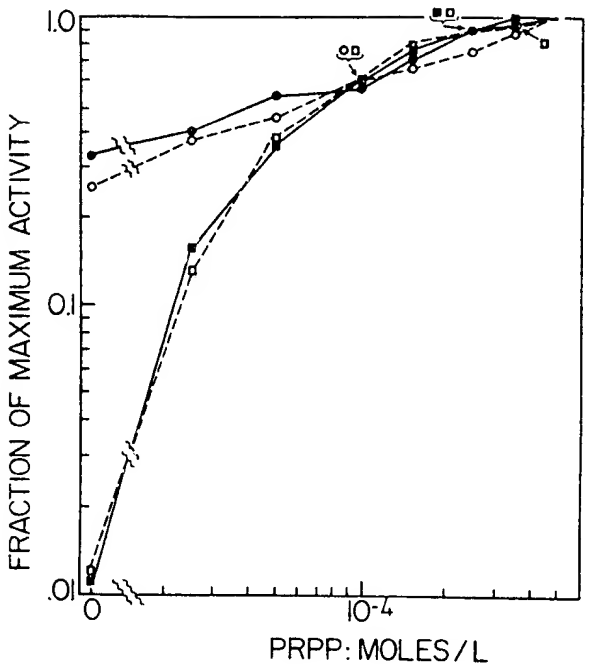
per gel for 45 min and then 2.5 mA per gel for 150 min. The enzyme zones in the gels were developed as described by Bakay and Nyhan (1971). The gels were fractionated in 1 or 2 mm sections with a Gilson Gel Fractionator (Model B-200). Using 60% ethanol as the fraction-collecting solution the fractions can be counted directly in a PPO-POPOP ethanol-toluene (5:9 v/v) scintillator (PPO, 6 g/l; POPOP 0.01 g/l). To avoid sticking of the gel fractions to the glass we used standard plastic vials (Packard) and counted in a liquid scintillation spectrometer (Packard, Tri-Carb Mod. 3380). Reproducible counts were obtained after the samples had been cooled down in the dark for at least 30 min



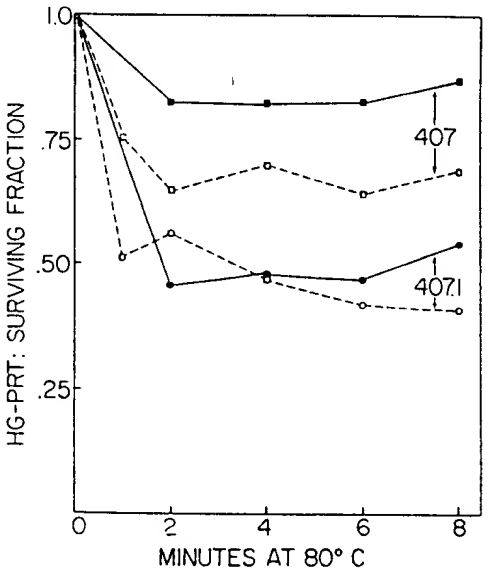
Experiment	Extract	μg Protein	Minutes at 37°	Maximum Activity (cpm)
I	407 : ■	12.5	45	678
	407.1 : ●	48	120	230
II	407 : □	19	60	1539
	407.1 : ○	44	120	401

Fig. 8. *HG-PRT activity as a function of PRPP concentration in dialyzed sonic extracts of mutant 407.1 and its parent, Strain 407*

(261, 353, 356, 371, 407 and 426) can be summarized as follows: I. Our technique reveals only one definite peak of HG-PRT activity in acrylamide gels; II. The relative sizes of the peaks found with different cell strains correspond to their relative specific enzyme activities, as determined with crude extracts or with the glass cylinder slide method. This indicates that the characteristic, graded reductions in HG-PRT activity that we observe in unfractionated cell preparations do not result from inhibitors that are separable from the enzyme protein during electrophoresis; III. The HG-PRT deficits associated with AG-resistance are specific, in the sense that A-PRT activities are quantitatively and electrophoretically unaltered; IV. The residual enzyme activities of all 12 mutants studied so far are electrophoretically indistinguishable from normal HG-PRT. We continue to investigate the possibility that the resolving power of our method is inadequate. One mutant (261.9) has been tested more extensively (gel lengths: 8.5, 12.0, 15.0 cm; total acrylamide concentrations: 6.0, 7.5, 9.0 and 2.66% and 5.0% of crosslinking agent) without any indication of an electrophoretic variation. Meanwhile, other differences between normal and mutant HG-PRT activities have been observed and will be illustrated here with mutant 407.1.

Table 6. *HG-PRT Activity in Mixed and Unmixed Extracts of Strain 407 and its Azaguanine-resistant Mutant 407.1.* Dialyzed sonic extracts were used. The reaction mixtures contained 6 μg of protein (No. 407), 48 μg of protein (No. 407.1) or a mixture of 6 μg of No. 407 and 42 μg of No. 407.1. Incubations were for 120 min

Extracts	PRPP		
	0 (cpm)	2×10^{-5} M (cpm)	3×10^{-5} M (cpm)
407	12	192	304
407.1	208	192	228
407 + 407.1	192	408	491



Experiment	Extract	μg Protein/ml during Heating	Minutes at 37°	Maximum Activity (cpm)
I	407 : ■	560	40	1000
	407.1 : ●	1800	120	438
II	407 : □	720	30	381
	407.1 : ○	1360	120	281

Fig. 9. *Thermal inactivation of HG-PRT activity in dialyzed sonic extracts of mutant 407.1 and its parent, Strain 407.* The extracts contained 0.1 M Tris · HCl, pH 7.4 and 10^{-3} M PRPP during heating at 80°. Residual HG-PRT activities were determined using the standard assay at 37°

PRPP Dependent HG-PRT Activity in Mutant 407.1. The surprising retention of most HG-PRT activity by undialyzed extracts of 407.1 in the absence of added PRPP prompted us to do experiments with dialyzed extracts. Fig. 8 depicts two experiments in which HG-PRT activity as a function of PRPP concentration was

compared using dialyzed extracts of parent Strain 407 and mutant 407.1. The mutant extracts retained 0.25 to 0.35 of maximum activity when PRPP was not added, while the residual activity of the parent strain fell to 0.02 of maximum. Mutant 407.2 did not differ significantly from Strain 407 in its PRPP dependence and the activity of all 3 strains was saturated above 10^{-3} M PRPP.

It was possible that factors promoting the breakdown or unavailability of PRPP in extracts of 407 and 407.2 were relatively minimized in extracts of 407.1. We determined the HG-PRT activity in mixed and unmixed extracts of 407 and 407.1 at 3 different concentrations of PRPP. Table 6 shows that the activity of the mixtures approximated the sum of the activities contributed by the 2 strains at each concentration tested and that in the absence of added PRPP the activity of the mixture corresponded to the activity of the unmixed 407.1 extract. These results do not support the notion that PRPP is made less available by a factor or factors in extracts of Strain 407; instead, they suggest that the mutation in 407.1 has altered the structure of HG-PRT in such a manner as to enhance its affinity for PRPP while reducing its overall activity. The finding that the HG-PRT in mutant 407.1 is relatively thermolabile supports this hypothesis. Fig. 9 illustrates two experiments that revealed this difference. It is noteworthy that the HG-PRT activities in cells cultured from a boy with the L-N syndrome have increased thermolability and *increased* dependence on PRPP as associated traits (McDonald and Kelley, 1971). We have observed qualitative alterations in the HG-PRT activities of crude extracts of mutants 261.9 (PRPP dependence, NaF sensitivity, altered pH optimum) and 407.2 (hypoxanthine dependence) but believe that the differences we have observed will best be demonstrated when the enzymes have been adequately purified and have now bent our efforts toward that goal.

Discussion

This report shows that mutation of some human genes can readily be studied using normal, diploid, human fibroblasts and standard cell culture technology. With one exception, the results we described were obtained with male, foreskin-derived cultures but we emphasize that similar results have been obtained here using cultures originating in females and from skin biopsies of other parts of the body.

It is noteworthy that the cloning efficiencies of the skin fibroblasts we used were high enough to permit their use at several important stages of mutation studies: I. The primary isolation of mutants by selection; II. The subclonal cultivation of mutant colonies. This was used to demonstrate that once AG-resistance appeared it did not disappear after many cell divisions in the absence of the selective agent and that the ability to utilize exogenous hypoxanthine for growth was an attribute of AG-resistant cells rather than of surviving, parental cells. III. The performance of a fluctuation test, in which mutant cells that originated during non-selective, clonal growth were recovered as AG-resistant colonies after subculture. This experiment showed that most, if not all, of the AG-resistant colonies we detected originated in preadaptive mutations before selection with AG. Upper and lower estimates of the rate of spontaneous mutation were obtained: 1.8×10^{-6} and 4.5×10^{-7} per cell generation, respectively.

Additional fluctuation tests will be performed with Strain 426, the one used here, and with other strains in order to get a representative estimate of the mutation rate. These estimates are needed as standards for evaluating mutagenic influences. The mean incidence of AG-resistant mutants in fibroblast cultures from 15 unrelated humans was 4.1×10^{-6}. This is the approximate baseline above which mutagenic effects will have to be detected. The capacity to detect modest increases will be necessary in order to avoid the use of intense mutagenic treatments that may be unrealistic with regard to *in vivo* conditions. Small increments above background will be meaningful, being relatively great in comparison to the rate of spontaneous mutation. For instance, we performed an experiment (Albertini and DeMars, 1972) in which the incidence of AG-resistant colonies after 150 r of X-rays was 19.8×10^{-6}, compared to a spontaneous incidence of 9.5×10^{-6}. The effect of the irradiation on cell viability was minor (the surviving fraction was 0.86) and the just doubled incidence of mutant cells might be given little weight. However, in relation to the estimated spontaneous mutation rate, 150 r induced between 3.3 and 13.2 times as many mutations as would have appeared without irradiation. If this response is representative of human genes, such slightly increased incidences would reflect serious elevations in the general mutation rate. Fortunately, the mutation rate for at least one other gene can now be studied for the purpose of comparison using human fibroblasts. We can identify strains of diploid cells in which mutations conferring resistance to 2,6 diaminopurine can be detected. Some of the mutants are deficient in adenine phosphoribosyl-transferase activity and are unable to utilize exogenous adenine for growth when purine biosynthesis is blocked (Rappaport and DeMars, to be published).

A mutation rate of 4.5×10^{-7} to 1.8×10^{-6} per diploid cell generation for a human gene is reasonable in relation to estimates of germinal mutation rates. Most estimates fall in the range of 10^{-5} to 10^{-4} per gamete per human generation, although the estimates for some genes are as low as 2×10^{-6} (Vogel, 1964). These incidences of mutant gametes must be divided by the number of gonial doublings per human generation in order to be comparable to our estimates. Since, for males, the number of gonial doublings is about 50, the commonest germinal mutation rates would range between 2×10^{-7} and 2×10^{-6} per gonial generation, a range that brackets our estimates of the rate of spontaneous mutation to AG-resistance. There are fewer gonial generations in females but the stored oocytes are subject to possible time-dependent mutation processes for about 45 years, which may result in the accumulation of mutations.

There is little information on comparative germinal rates of spontaneous mutation males and females but we have the beginning of such information for somatic cells. Table 1 shows that most spontaneous incidences of AG-resistant mutants in cultures of male cells range between 10^{-6} and 10^{-5} and that one mutant, 261.9, was recovered from 10^6 cells of female Strain 261. In a different experiment with a second strain of female cells (No. 129) 11 mutant colonies were found among 1.92×10^6 cells. The incidence was 5.7×10^{-6}. In addition, three experiments with these same female strains indicate that they are about as susceptible to the mutagenic effects of X-rays and MNNG as male cells (Kahan and DeMars). Systematic studies of the relative mutation rates in male and female cells are planned but their apparent equality with regard to AG-resistance indicates that the mutations

are dominant or that they are recessive and occur on the single X chromosome of male cells or the single-active-X chromosome of female cells.

The range of variation in the spontaneous incidences of mutants in human cell cultures contrasts with the relatively greater variation observed in cultures of diploid Chinese hamster cells. The spontaneous incidence has ranged from about 10^{-3} (Bridges and Huckle, 1970) to 10^{-6} (Chu *et al.*, 1969) in different laboratories. The two published estimates of the rate of spontaneous mutation to AG-resistance in these cells also differ greatly although the cultures used had a common origin: 2×10^{-5} (Harris, 1971) and 1.6×10^{-8} (Chu *et al.*, 1969) per cell generation. Since our results with human cells are, for the moment, unique and those with Chinese hamster cells greatly discordant there is no point in attempting a detailed comparison at this time.

The changes that confer AG-resistance on diploid human fibroblasts remain undefined for the most part. We think they are stable hereditary changes because resistance persists after many cell generations in the absence of the selective agent. The changes are diverse, since we recovered mutants with distinctly different HG-PRT activities and growth properties. Two general types were observed: Class I mutants resemble cells from L-N boys in having very low HG-PRT activity, an inability to use hypoxanthine for growth and relatively great resistance to AG at concentrations between 10^{-4} M and 10^{-3} M. Mutant 407.1 exemplifies this class of mutants, which was recovered only once among at least 10 independent mutants that were characterized. Class II mutants have apparent HG-PRT activities that range from normal (e.g. 261.9) to L-N amounts (e.g. 356.8, 371.1), they are able to use hypoxanthine for growth and are partially inhibited by AG at concentrations of 10^{-4} M or more. Excepting 261.9, all AG-resistant mutants that were tested had distinct reductions in HG-PRT activity (Fig. 3). There is no evidence that the selective conditions used eliminated a large class of mutants that might have been recovered at lower concentrations of AG. One experiment with Strain 407 yielded 20 resistant colonies from 2×10^{6} cells selected against at 8×10^{-6} M AG and 8 colonies from 10^{6} cells selected against at 3×10^{-6} M AG.

Two kinds of evidence favor an interpretation of our results in terms of genetic rather than simply phenotypic changes: I. The incidence of variants can be increased by the known mutagens, X-rays (Albertini and DeMars, 1972) and MNNG (Felix *et al.*, 1972) in a dose-dependent manner and the induced mutants have the same spectrum of phenotypic characteristics as the spontaneous mutants described above. II. The properties of some of the spontaneous mutants resemble the properties of cells from humans with inherited mutant genes. The best evidence pertains to Class I mutant 407.1, which resembles L-N cells. The scant HG-PRT activity of this mutant was more thermolabile and less dependent on PRPP than the normal enzyme activity. In contrast, Class II mutant 261.9 could use hypoxanthine for growth as well as normal cells and had an apparently normal amount of HG-PRT activity. We presented evidence that these associated traits, too, were found in a standard mutant strain, No. 260, derived from a male with an inherited X-chromosomal mutant gene that specified qualitatively altered HG-PRT activity. This argument by analogy is not conclusive, especially for the great majority of Class II mutants we studied. Their ubiquity agrees with the finding that many leukemias that become resistant to 6-mercaptopurine are not markedly deficient

in HG-PRT (Davidson and Winter, 1964) but we wonder why more males with this type of mutant gene haven't been described. They should be more common than boys with the Lesch-Nyhan syndrome. Perhaps they are to be found among the males who have gout associated with partial reductions in HG-PRT activity (Kelley *et al.*, 1969). A survey of AG-resistance and HAT-growth of cells cultured from such males should be undertaken.

We are trying to determine the detailed nature of the changes that occur in our AG^r mutants. One is a straightforward search for structural differences in their HG-PRT proteins. Electrophoretic differences have not been detected so far but preliminary observations of altered kinetic behavior in some of these mutants are promising enough to warrant attempts to partially purify the enzymes. The finite propagability of diploid fibroblasts is a real impediment to this kind of study but we emphasize that mutant clones have often been grown to populations of 10^8—10^9 cells i.e. about 0.1—1.0 g of cell protein. Scaled down procedures should permit the recovery of enough purified HG-PRT activity for critical characterization.

The second approach is to prepare hybrids of hypoxanthine-utilizing human cell mutants with an amorphic, non reverting, HG-PRT-deficient mouse cell mutant. The hybrids can be isolated by selection in CS-F10-HAT because they are morphologically distinctive and can outgrow the human cell parent. Weiss and Green (1967) first demonstrated that human chromosomes are eliminated from human mouse hybrid cells until only those remain that are necessary for selective growth. Nabholz *et al.* (1969) have shown that crosses between normal human cells and HG-PRT-deficient mouse cells yield HAT-selected hybrids in which the human X is specifically retained. The karyotypic and enzymatic properties of the hybrids we are preparing should yield information about the specific human chromosome or chromosomes that are responsible for the associated traits of reduced HG-PRT activity and ability to utilize hypoxanthine.

The ability of most AG-resistant mutants to grow in CS-F10-HAT has had two undesirable consequences. It prevents the elimination of preexisting AG-resistant mutants prior to the determination of a mutation rate, and, thereby, decreases sensitivity in detecting mutagenic effects. This can be partially circumvented by pre-screening cell populations for those that have minimum incidences of mutants, such as Strain 370 (Table 1). However, the gain is slight and is likely to be variable from one population sample to another of the same strain because the population size needed for detecting modest mutagenic effects is about the reciprocal of the spontaneous mutation rate i.e. there is a high probability that any cell population used for a small-effect mutagenesis experiment will contain some spontaneous mutants. However, the background of spontaneous mutants is low enough so that doublings can be reliably demonstrated in practical experiments and this is good enough for detecting substantial increases above the spontaneous mutation rate. A second way of circumventing the problem is to eliminate the preexisting non-HAT-growable mutants and then to select only for Class I mutants in determining mutation rates. Our results suggest that L-N cells (e.g. Strain 284) and L-N-like mutants (e.g. 407.1) are little inhibited at concentrations of AG above 10^{-4} M, which significantly impair the growth of Class II mutants, such as Strains 260, 261.9 and 407.2 (Fig. 4). A possible disadvantage of this apparently neater selective system is its emphasis on a minor fraction of the mutational potentiality for AG-resistance.

Specific selection for Class I mutants at high concentrations of AG might solve the second problem resulting from the ubiquity of Class II mutants. In principle, selection for cells in which constitutive derepression of the inactive X chromosome has occurred can be practiced in populations of female cells that are heterozygous for L-N mutant alleles. There are two phenotypic classes of heterozygous cells. Using previously proposed symbolism (DeMars, 1971), the "normal" cell class is $[hpt^+]_a/[hpt^-]_i$, the "mutant" class is $[hpt^+]_i/[hpt^-]_a$. Alleles in brackets with subscript "a" are on the active X and determine the phenotype of the cell, while alleles in brackets marked "i" are on the inactive X. "Mutant" cells are AG-resistant and unable to grow in HAT medium. Derepression of the hpt^+ allele in "mutant" cells could then be detected by selection in HAT medium. We proposed that suitable populations of "mutant" cells could be produced simply by applying AG-selection to uncloned cultures of heterozygous cells. It is clear now that the cell-inocula used for such selection will have to be small enough to certainly exclude Class II mutants of "normal" cells, for these would be selected out in AG-medium, along with the heterozygous cells, and could then simulate "mutant" cells with derepressed X chromosomes by virtue of their ability to grow in HAT. Selection at AG concentrations above 10^{-4} M might further insure that Class II mutants are not present among the "mutant" cells used in such experiments.

Mutant 261.9 is a female Class II mutant. It was isolated from a culture of cells that were heterozygous for gpd^{B+} and gpd^{ts} alleles, the latter determining the production of a temperature-sensitive form of G6PD (DeMars, 1968). If the AG-resistance mutation occurred on the active X chromosome, mutant 261.9 is doubly heterozygous at X-chromosomal loci. Similar mutants have been isolated from female strains that are heterozygous for other G6PD alleles. The use of double heterozygotes in studying X-chromosome derepression has been discussed previously (DeMars, 1971). It is encouraging that one can so easily produce them in the laboratory instead of having to rely on rare humans with the desired genotypes. This means that the process of mutation can not only be studied for its own sake with diploid human cells but can now serve as a laboratory tool for many kinds of genetic experiments that previously could not be performed.

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Discussion

Vogel: Did you compare cells of patients with Lesch-Nyhan syndrome with those of your artificial mutants?

DeMars: This is a very important point. We had a boy who did not show the Lesch-Nyhan syndrome, who, however, produced as much uric acid as Lesch-Nyhan patients do. We studied the cells from this family and found that the females had two phenotypic populations of cells. It turned out that the enzyme of this family — using the criteria which I have described — is perfectly as 261.9. This boy was paradoxial because he had a normal amount of HGPRT activity in his blood and his fibroblasts; but his enzyme was purine sensitive and PRDP dependant, and we have the information that it is transmitted to the females in the family. The correspondence between the *in vitro* results and the *in vivo* results is fairly good.

Vogel: Is it not possible to investigate the temperature dependance of the mutation rate in your system? Or do you depend on 37°?

DeMars: One can get a range of about 7 or 8 degrees, that is between 32 and 39°, to work in. My feeling is that this system is also going to be useful for other investigations, for instance the study of mutations in resting cells. The important point about this method is that any strain of fibroblasts can give these mutants.

Klamerth: Did you check the migration differences between the mutants in gel electrophoresis?

DeMars: So far we did not find any difference in all our mutants using acrylamide gel for electrophoresis and a variety of buffers under a variety of conditions. They all migrate perfectly as the normal enzyme does. But we now started electrofocusing investigations.

Holliday: How do you overcome the problem of cell density when you select these mutants — because the mutants are not expressed when the cells are crowded?

DeMars: In this system phenotypicaly mutant cells become normal phenocopies when they are in contact with phenotypical normaly cells and that means that they do not survive the azaguanine selection. The only way to circumvent this is to work with low density populations.